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TITLE: Testing ER-Beta Agonist Synergy with B7-H1 and mTOR Inhibitors as Novel and Effective Treatments for Ovarian Cancer

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14. ABSTRACT

αB7-H1 immunotherapy and ERβ agonist therapy as single agents were ineffective to treat large tumor volume ovarian cancer in the ID8 mouse model. Using ERβ KO and B7-H1 KO mice we showed that poor treatment responses were not due to host B7-H1 or ERβ signals. The combination of αB7-H1 plus the ERβ agonist LY was modestly effective in large tumor volume disease in the model, but the LY caused some toxicities seen as cold paws that could be from compromised blood flow. We found that tumor B7-H1 signals can alter ERβ signals, seen as reduced ERβ-dependent VEGF production, which could play a role in treatment efficacy. Work to investigate treatment effects in lower tumor burden and with addition of mTOR inhibitors is underway.

15. SUBJECT TERMS
None listed

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INTRODUCTION

Ovarian cancer (OC) is the fifth leading cause of cancer death in US women and is the top gynecologic cancer killer. The ACS estimates ~21,980 new OC cases and ~14,270 deaths in the US in 2014. Most patients fail front-line therapy and will die of their disease. More effective treatments for OC are thus urgently needed.

ERβ in OC. The biological effects of estrogens (E2) are mediated through their cognate receptors: estrogen receptor (ER)α and ERβ[1]. ERβ functions differ from ERα, and ERβ functions as a tissue-specific tumor suppressor with anti-proliferative actions[2]. Despite ER expression in 67% of OC, anti-E2 therapy has limited success and the benefit of hormonal therapy has not been systematically studied[3]. Even through E2 functions through ERβ, E2 as OC therapy has limited therapeutic application due to the suspected role of E2 in the etiology of OC. *Emerging evidence suggests that OC tumor cells express ERβ. ERβ could act as a tumor suppressor, lack of mechanistic insights has hindered drug development for OC use.*

ERβ agonists. ERα and ERβ are structurally similar, but their ligand-binding domains differ enough to be selective for unique ligands. Recently, a number of selective ERβ agonists have been identified and are being investigated for therapeutic use[2]. Liquiritigenin (Liq) is a novel, highly selective ERβ agonist[4] designed to treat menopausal vasomotor symptoms. LY500307 is a potent (EC_{50} 0.66 nM) and efficacious ERβ agonist with ~30 fold selectivity over the ERα receptor and >100 fold selectivity against non-related receptors[5]. LY500307 showed an excellent preclinical and phase 1 profile in clinical trials and is currently in phase II clinical trials for schizophrenia. *Availability of these unique ligands that act through ERβ provides a novel therapeutic opportunity to target ERβ for suppression of OC, which we will use here.*

mTOR. The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that belongs to the PI3K-related kinase family[6]. mTOR plays an important role in cell growth, proliferation, autophagy, ribosomal biogenesis, development and aging[7]. mTOR exists as two complexes: the mTOR/mLST8/Raptor-containing mTORC1 complex and the mTOR/mLST8/Rictor-containing mTORC2 complex[8,9]. mTORC1 phosphorylates and activates downstream signaling components such as S6K and 4E-BP1, both of which are involved in protein translation. mTORC2 associates with ribosomes and facilitates their activation[9]. mTORC2 also phosphorylates Akt/PKB and SGK1[8,10]. The mTOR/PI3K/Akt pathway is commonly altered/activated in OC[11] and mTOR inhibitors are currently being evaluated in phase I/II trials for treatment of OC. *We discovered unexpected crosstalk of ERβ signaling with mTOR that will be tested as a treatment modality to enhance ERβ tumor suppressor functions.*

B7-H1 (CD274, PD-L1), a member of the B7-H (B7 homology) family that is an immune co-signaling molecule[12]. It controls maternal-fetal tolerance[13] by influencing regulatory T cell (Treg)

function[14], facilitates induced Treg generation[15], governs gut tolerance[16] and affects autoimmunity in animal models[17-19]. It is now also under intense investigation as a novel anti-cancer immunotherapy target[12]. We showed that B7-H1 generates Tregs in human OC and hinders anti-OC immunity[20]. B7-H1 on OC tumor cells also inhibits anti-cancer immunity[21]. We found that female B7-H1$^{-/-}$ mice (lacking B7-H1) survived challenge with ID8 OC cells better than WT females[22]. Using available agents to manipulate B7-H1, which is highly expressed in most OC, we found that ERβ signals can be altered through mTOR signals to reduce OC tumor growth.

KEYWORDS
Ovarian cancer, mTOR, estrogen receptor beta, PD-L1, combination therapy

ACCOMPLISHMENTS

1.1 Treat WT mice bearing ID8 tumors with ERβ agonist (LY) alone vs control This was accomplished. The specific ERβ agonist LY500307 (LY) had no effect on ID8 tumors, or made them grow slightly faster versus controls. We also used ERβ KO mice to see if ERβ signals on immune cells had a deleterious effect, to explain the disconnect between good *in vitro* ERβ agonist tumor suppression but lack of *in vivo* efficacy. However, LY also had no effect on tumor growth when ID8 was challenged into ERβ KO mice.	1-8
1.2 Treat WT mice bearing ID8 tumors with mTORC1 inhibitor (rapamycin) ± LY + controls. The mTORC1 inhibitor rapamycin had no significant effect on tumor growth. Combination with LY is now understudy.	3-12
1.3 Treat WT mice bearing ID8 tumors with mTORC1/2 inhibitor ZD ± LY + controls. This was deferred to year 2 (still on time) as we focused on rapamycin, ERβ KO and B7-H1 KO studies.	5-15
Milestone(s) Achieved: We have identified the lack of utility of an mTORC1 inhibitor and the lack of αB7-H1 each as single agents when given one week after tumor challenge.	18
2.1. Test αB7-H1 + ERβ agonists in WT mice bearing ID8 tumors The B7-H1 effect was initially assessed by challenging ID8 into B7-H1 KO mice. The effect of the ERβ-specific agonist LY was not significantly affected in B7-H1 KO versus WT mice. αB7-H1 alone was tested and did not significantly alter tumor growth when given 7 days after tumor challenge. When we added αB7-H1 to LY, we got a borderline significant improvement in survival in ID8 challenge versus αB7-H1 alone and against LY alone (p=0.05). We are now testing giving αB7-H1 at earlier time points after tumor challenge (2-5 days) and once we establish an effective time to give, we will test the combination with LY. The other issues is that the LY when given with αB7-H1 for extended	9-17

periods (2-3 weeks) causes some mouse toxicities as seen by cold and whitish paws. We will test using lower LY doses in future experiments to see if treatment effects can be improved by reducing toxicities. If no αB7-H1 treatment effect alone is defined, we will use the earliest time point (2 days after tumor challenge) and assess LY effects, which still could be observed even if αB7-H1 alone is not effective.	
2.2. Test αB7-H1 + ERβ agonists + mTOR inhibitors in WT mice bearing ID8 tumors We are awaiting data from **task 2.1** to test using all three agents together. This task is still on time.	9-17
Milestone(s) Achieved: We have identified potential utility of an estrogen receptor β agonist (LY) plus αB7-H1. We have eliminated effects on host B7-H1 or host ERβ as reasons for lack of better single agent efficacy.	18
Aim 3 Identify ERβ/B7-H1 effects in human OC cells in WT mice bearing ID8 tumors	
3.1 Test the effect of ERβ/B7-H1 cross talk in human OC cells in vitro We showed that the human ovarian cancer cell lines ES2, OVCAR3 and SKOV3 are all B7-H1 positive by flow cytometry, with ES2 also validated by Western blot. We used shRNA to create B7-H1^{lo} ES2 cells. We are now testing effects of ERβ agonist on cell proliferation and signaling. This was to be done by month 12 and is thus slightly delayed, but is progressing and will be completed before the end of the funding period.	1-12
3.2. Identify specific mechanisms for B7-H1-mediated regulation of ERβ in human tumors This work is for year 2, but in initial studies, we treated control or B7-H1^{lo} (from shRNA) ID8 cells ± LY to agonize the estrogen receptor beta. We used RNA-seq to evaluate differential gene expression. PD-L1 status or LY treatment had no effect on expression of the ERβ target molecules estrogen receptor alpha, estrogen receptor beta, progesterone receptor, GRP30 and several additional genes. However, tumor PD-L1 appeared to drive LY (estrogen receptor beta)-mediated VEGF expression as it was significantly lower in PD-L1^{lo} tumors versus control tumors but only with LY treatment.	13-24
3.3. Identify mechanisms for B7-H1-mediated mTOR regulation in human OC in vitro This work is now beginning in year 2.	13-24
Milestone(s) Achieved: We have identified specific elements of B7-H1 effects on ERβ signals using mouse tumor cells. These have formed the basis for the work in human cells proposed for year 2.	

What opportunities for training and professional development has the project provided?
Nothing to Report

How were the results disseminated to communities of interest?
Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?
We are now testing αB7-H1 treatment effects at smaller tumor burdens. Further, we have seen potential benefits by adding the ERβ agonist LY. We will test adding the mTOR inhibitor to αB7-H1 or LY assess for additional clinical benefits.

IMPACT
What was the impact on the development of the principal discipline(s) of the project?
We have furthered understanding of effects of B7-H1 on ERβ signals and treatment effects. We have defined potential toxicities of ERβ agonists clinically and are working to mitigate them while maintaining clinical efficacy.

What was the impact on other disciplines?
Our data on B7-H1 and ERβ effects will inform others testing immunotherapy or ERβ agonists for other diseases such as breast cancer.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

CHANGES/PROBLEMS
Changes in approach and reasons for change
We added studies of B7-H1^{lo} tumor cells to complement αB7-H1 blocking data, as the cells became available to us through other work done in the lab. We added RNA-seq to evaluate cross talk effects, as the technology is powerful and is now available to us. We added studies of ERβ KO mice and B7-H1 KO mice to assess host signals as we did not see expected beneficial effects of ERβ agonists and αB7-H1 as expected.

Actual or anticipated problems or delays and actions or plans to resolve them
In **1.1** we did not see the expected benefit of using an ERβ agonist *in vivo* to treat ID8 challenge. We suspected that the ERβ agonist could have detrimental effects on anti-tumor immunity but failed also to see benefits using ERβ KO mice. As we saw potential benefit when αB7-H1 was added, this issue can be potentially mitigated. Further, by adding the mTOR inhibitor we could see additional clinical benefits.

In **1.2** we focused efforts on defining host B7-H1 and ERβ signals and this slightly delayed testing mTOR inhibitor combinations with ERβ agonists *in vivo*. We now report that adding the ERβ agonist LY to αB7-H1 improved tumor control in ID8agg aggressive ovarian cancer (**Fig. 1,** on the next page).

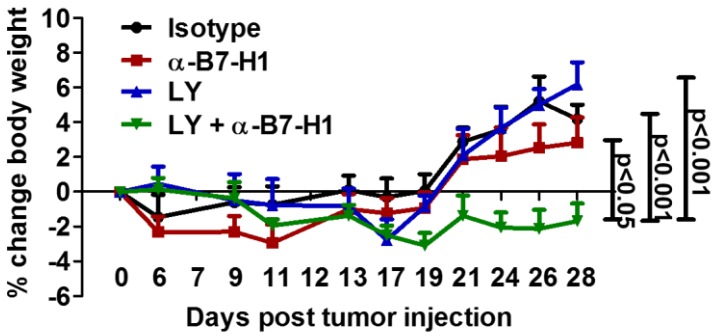

Fig. 1 The ERβ agonist LY improves tumor control in ID8agg tumor challenge. Groups of N=7 mice challenged with 4 x 10^6 ID8agg cells by intraperitoneal injection and treated with LY, αB7-H1, both or controls, as per the protocol. Body weight is ascites, an accepted measure of tumor growth in this model.

In **2.1** we have not seen the expected beneficial effect of αB7-H1 as a single agent to treat ID8 tumor challenge, but we think this could be from waiting until the tumor burden is too high. We are now testing αB7-H1 treatment effects at smaller tumor burdens. Further, we have seen potential benefits by adding the ERβ agonist LY, as shown above in **Fig. 1**.

In **2.2** we had to wait for *in vivo* LY data and then had to deal with unexpected toxicities of LY to combine with αB7-H1. That issue will be resolved by testing lower LY doses, which is underway, and we will then proceed to combine LY with αB7-H1, which is slightly delayed but which will be finished within this funding period.

Changes that had a significant impact on expenditures
We identified unexpected LY toxicity which requires additional *in vivo* toxicity testing. This will use more LY and more mice than originally expected, but we expect that we can nonetheless accomplish all scientific goals as originally proposed, unless identifying a better LY regimen takes longer than anticipated. Lack of efficacy of αB7-H1 and of ERβ agonists as single agents *in vivo* necessitated mechanistic studies using ERβ KO and B7-H1 KO mice, but these expenses were modest and can be absorbed into current work.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
None.

Significant changes in use or care of human subjects
Not applicable.

Significant changes in use or care of vertebrate animals
We identified unexpected LY toxicity which requires additional *in vivo* toxicity testing and produced unexpected animal toxicities. We are monitoring mice daily, and continue to use previously established criteria to determine if suffering is undue and requires sacrifice, which has thus far not happened.

Significant changes in use of biohazards and/or select agents
Not applicable.

PRODUCTS
Nothing to report

PARTICIPANTS and other collaborating organizations

What individuals have worked on the project?
Name: Tyler Curiel
Project role: PI
Research identifier: TCURIEL
Nearest person month worked: 1
Contribution to project: Provided overall supervision, interpreted data, provided troubleshooting guidance, managed budget, wrote progress report

Name: Ratna Vadlamudi
Project role: co-investigator
Research identifier: VADLAMUDI
Nearest person month worked: 0.5
Contribution to project: provided expertise on ERβ signals, provided guidance on LY dosing and adjustments for toxicity, helped interpret data, helped write progress report

Name: Harshita Gupta
Project role: post-doctoral fellow
Research identifier: None
Nearest person month worked: 6
Contribution to project: did hands on *in vitro* and mouse work for tumor challenges, treatments and data analysis and graphing

Name: Shunhua Lao
Project role: technician
Research identifier: None
Nearest person month worked: 2
Contribution to project: maintained mice, gave treatments, monitored for toxicities and tumor growth, helped stock lab supplies, helped process tissues for *ex vivo* work

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to Report.

Special reporting requirements
None.

Appendices
None.